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To:
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NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year) 19 April 2001 (19.04.01)		
Applicant's or agent's file reference 5958	IMPORTANT NOTICE	
International application No. PCT/DK00/00577	International filing date (day/month/year) 12 October 2000 (12.10.00)	Priority date (day/month/year) 14 October 1999 (14.10.99)
Applicant NOVOZYMES A/S		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
AU,KP,KR

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2. The following designated Offices have waived the requirement for such a communication at this time:
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The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on 19 April 2001 (19.04.01) under No. WO 01/27251

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If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

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The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

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(54) Title: LYSOPHOSPHOLIPASE FROM ASPERGILLUS

(57) Abstract: The inventors have isolated lysophospholipases from *Aspergillus* (*A. niger* and *A. oryzae*) having molecular masses of about 68 kDa and amino acid sequences of 600-604 amino acid residues. The novel lysophospholipases have only a limited homology to known amino acid sequences. The inventors also isolated genes encoding the novel enzymes and cloned them into *E. coli* strains.

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LYSOPHOSPHOLIPASE FROM ASPERGILLUS

FIELD OF THE INVENTION

The present invention relates to lysophospholipases (LPL), methods of using and producing them, as well as nucleic acid sequences encoding them.

BACKGROUND OF THE INVENTION

Lysophospholipases (EC 3.1.1.5) are enzymes that can hydrolyze 2-lysophospholids to release fatty acid. They are known to be useful, e.g., for improving the filterability of an aqueous solution containing a starch hydrolysate, particularly a wheat starch hydrolysate (EP 219,269).

N. Masuda et al., Eur. J. Biochem., 202, 783-787 (1991) describe an LPL from *Penicillium notatum* as a glycoprotein having a molecular mass of 95 kDa and a published amino acid sequence of 603 amino acid residues. WO 98/31790 and EP 808,903 describe LPL from *Aspergillus foetidus* and *Aspergillus niger*, each having a molecular mass of 36 kDa and an amino acid sequence of 270 amino acids.

JP-A 10-155493 describes a phospholipase A1 from *Aspergillus oryzae*. The mature protein has 269 amino acids.

SUMMARY OF THE INVENTION

The inventors have isolated lysophospholipases from *Aspergillus* (*A. niger* and *A. oryzae*) having molecular masses of about 68 kDa and amino acid sequences of 600-604 amino acid residues. The novel lysophospholipases have only a limited homology to known amino acid sequences. The inventors also isolated genes encoding the novel enzymes and cloned them into *E. coli* strains.

Accordingly, the invention provides a lysophospholipase which may be a polypeptide having an amino acid sequence as the mature peptide shown in one of the following or which can be obtained therefrom by substitution, deletion, and/or insertion of one or more amino acids, particularly by deletion of 25-35 amino acids at the C-terminal:

SEQ ID NO: 2 (hereinafter denoted *A. niger* LLPL-1),
SEQ ID NO: 4 (hereinafter denoted *A. niger* LLPL-2),
SEQ ID NO: 6 (hereinafter denoted *A. oryzae* LLPL-1), or
SEQ ID NO: 8 (hereinafter denoted *A. oryzae* LLPL-2).

Further, the lysophospholipase of the invention may be a polypeptide encoded by the lysophospholipase encoding part of the DNA sequence cloned into a residues can be aligned with the mature *A. oryzae* LLPL-2 of the invention (604 amino acids) with a homology of 79 %.

DETAILED DESCRIPTION OF THE INVENTION

Genomic DNA source

Lysophospholipases of the invention may be derived from strains of *Aspergillus*, particularly strains of *A. niger* and *A. oryzae*, using probes designed on the basis of the DNA sequences in this specification.

Strains of *Escherichia coli* containing genes encoding lysophospholipase were deposited by the inventors under the terms of the Budapest Treaty with the DSMZ - Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig DE as follows:

Source organism	Designation of lysophospholipase	Accession number	Date deposited
A. niger	LLPL-1	DSM 13003	18 August 1999
A. niger	LLPL-2	DSM 13004	18 August 1999
A. oryzae	LLPL-1	DSM 13082	8 October 1999
A. oryzae	LLPL-2	DSM 13083	8 October 1999

C-terminal deletion

The lysophospholipase may be derived from the mature peptide shown in SEQ ID NOS: 2, 4, 6 or 8 by deletion at the C-terminal to remove the ω site residue while preserving the lysophospholipase activity. The ω site residue is described in Yoda et al. Biosci. Biotechnol. Biochem. 64, 142-148, 2000, e.g. S577 of SEQ ID NO: 4. Thus, the C-terminal deletion may particularly consist of 25-35 amino acid residues.

A lysophospholipase with a C-terminal deletion may particularly be produced by expression in a strain of *A. oryzae*.

Properties of lysophospholipase

The lysophospholipase of the invention is able to hydrolyze fatty acyl groups in lysophospholipid such as lyso-lecithin (Enzyme Nomenclature EC 3.1.1.5). It may also be able to release fatty acids from intact phospholipid (e.g. lecithin).

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a x-ray film.

Alignment and homology

The lysophospholipase and the nucleotide sequence of the invention preferably have homologies to the disclosed sequences of at least 80 %, particularly at least 90 % or at least 95 %, e.g. at least 98 %.

For purposes of the present invention, alignments of sequences and calculation of homology scores were done using a full Smith-Waterman alignment, useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in a gap is -2 for proteins and -4 for DNA. Alignment is from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98). Multiple alignments of protein sequences were done using "ClustalW" (Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22:4673-4680). Multiple alignment of DNA sequences are done using the protein alignment as a template, replacing the amino acids with the corresponding codon from the DNA sequence.

Lysophospholipase activity (LLU)

Lysophospholipase activity is measured using egg yolk L-α-lysolecithin as the substrate with a NEFA C assay kit.

20 µl of sample is mixed with 100 µl of 20 mM sodium acetate buffer (pH 4.5) and 100 µl of 1% L-α-lysolecithin solution, and incubated at 55°C for 20 min. After 20 min, the reaction mixture is transferred to the tube containing 30 µl of Solution A in NEFA kit preheated at 37°C. After 10 min incubation at 37°C, 600 µl of Solution B in NEFA kit is added to the reaction mixture and incubated at 37°C for 10 min. Activity is measured at 555 nm on a spectrophotometer. One unit of lysophospholipase activity (1 LLU) is defined as the amount of enzyme that can increase the A550 of 0.01 per minute at 55°C.

John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990.

Enzymes

Enzymes for DNA manipulations (*e.g.* restriction endonucleases, ligases etc.) are obtainable from New England Biolabs, Inc. and were used according to the manufacturer's instructions.

Plasmids/vectors

pT7Blue (Invitrogen, Netherlands)

pUC19 (Genbank Accession #: X02514)

pYES 2.0 (Invitrogen, USA).

Microbial strains

E. coli JM109 (TOYOBO, Japan)

E. coli DH12α (GIBCO BRL, Life Technologies, USA)

Aspergillus oryzae strain IFO 4177 is available from Institute for Fermentation, Osaka (IFO) Culture Collection of Microorganisms, 17-85, Juso-honmachi, 2-chome, Yodogawa-ku, Osaka 532-8686, Japan.

A. oryzae BECh-2 is described in Danish patent application PA 1999 01726. It is a mutant of JaL 228 (described in WO 98/12300) which is a mutant of IFO 4177.

Reagents

NEFA test kit (Wako, Japan)

L-α-lysolecithin (Sigma, USA).

Media and reagents

Cove: 342.3 g/L Sucrose, 20 ml/L COVE salt solution, 10mM Acetamide, 30 g/L noble agar.

Cove-2: 30 g/L Sucrose, 20 ml/L COVE salt solution, 10mM, Acetamide, 30 g/L noble agar.

Cove salt solution: per liter 26 g KCl, 26 g MgSO4-7aq, 76 g KH2PO4, 50ml Cove trace metals.

Cove trace metals: per liter 0.04 g NaB4O7-10aq, 0.4 g CuSO4-5aq, 1.2 g FeSO4-7aq, 0.7 g MnSO4-aq, 0.7 g Na2MoO2-2aq, 0.7 g ZnSO4-7aq.

AMG trace metals: per liter 14.3 g ZnSO4-7aq, 2.5 g CuSO4-5aq, 0.5 g NiCl2, 13.8 g FeSO4, 8.5 g MnSO4, 3.0 g citric acid.

YPG: 4 g/L Yeast extract, 1 g/L KH2PO4, 0.5 g/L MgSO4-7aq, 5 g/L Glucose, pH 6.0.

STC: 0.8 M Sorbitol, 25 mM Tris pH 8, 25 mM CaCl2.

Step	Temperature	Time
1	94°C	2 min
2	92°C	1 min
3	55°C	1 min
4	72°C	1 min
5	72°C	10 min
6	4°C	forever

Steps 2 to 4 were repeated 30 times.

The expected size, 1.0 kb fragment was gel-purified with QIA gel extraction kit (Qiagen, Germany) and ligated into a pT7Blue vector with ligation high (TOYOBO, Japan). The ligation mixture was transformed into *E. coli* JM109. The resultant plasmid (pHUda94) was sequenced and compared to the *Penicillium* lysophospholipase, showing that a clone encodes the internal part of the lysophospholipase.

Cloning of llpl-1 gene

In order to clone the missing part of the lysophospholipase gene, a genomic restriction map was constructed by using the PCR fragment as probes to a Southern blot of *Aspergillus niger* DNA digested with seven restriction enzymes, separately and probed with 1.0 kb fragment encoding partial lysophospholipase from pHUda94.

A hybridized 4-6 kb SphI fragment was selected for a llpl-1 gene subclone.

For construction of a partial genomic library of *Aspergillus niger*, the genomic DNA was digested with SphI and run on a 0.7 % agarose gel. DNA with a size between 4 to 6 kb was purified and cloned into pUC19 pretreated SphI and BAP (Bacterial alkaline phosphatase). The sphI sub-library was made by transforming the ligated clones into *E. coli* DH12α cells. Colonies were grown on Hybond-N+ membranes (Amersham Pharmacia Biotech, Japan) and hybridized to DIG-labelled (Non-radio isotope) 1.0 kb fragment from pHUda94.

Positive colonies were picked up and their inserts were checked by PCR. Plasmids from selected colonies were prepared and sequenced revealing 5 kb SphI fragment were containing whole llpl-1 gene.

Expression of llpl-1 gene in *Aspergillus oryzae*.

The coding region of the LLPL-1 gene was amplified from genomic DNA of an *Aspergillus niger* strain by PCR with the primers HU188 (SEQ ID NO: 11) and HU189 (SEQ ID NO: 12) which included a EcoRV and a XhoI restriction enzyme site, respectively.

creased lysophospholipase activity in supernatants and the presence of increased lysophospholipase activity in cell free extracts.

Strain	Yield (supernatant) Relative activity	Yield (Cell fraction) Relative activity
BECh-2	1.0	1.0
LP3	1.0	4.5
	1.0	4.0
LP8	1.0	6.5
	1.0	5.5

Example 2: Cloning and expression of LLPL-2 gene from *A. niger*

Preparation of a llp2 probe

The same strain of *Aspergillus niger* as in Example 1 was used as a genomic DNA supplier.

PCR reactions on *Aspergillus niger* genomic DNA was done with the primers HU212 (SEQ ID NO: 13) and HU213 (SEQ ID NO: 14) designed based upon amino acid sequences from purified lysophospholipase from AMG 400L (described in Example 4).

Reaction components (1 ng /µl of genomic DNA, 250 mM dNTP each, primer 250 nM each, 0.1 U/ µl in Taq polymerase in 1X buffer (Roche Diagnostics, Japan)) were mixed and submitted for PCR under the following conditions.

Step	Temperature	Time
1	94°C	2 min
2	92°C	1 min
3	50°C	1 min
4	72°C	1 min
5	72°C	10 min
6	4°C	forever

Steps 2 to 4 were repeated 30 times.

The expected size, 0.6 kb fragment was gel-purified with QIA gel extraction kit (Qiagen, Germany) and ligated into a pT7Blue vector with ligation high (TOYOBO, Japan). The ligation mixture was transformed into *E. coli* JM109. The resultant plasmid (pHUda114) was sequenced and compared to the *Penicillium* lysophospholipase, showing that a clone encodes the internal part of the lysophospholipase.

JM109. The resultant plasmid (pLLPL2) was sequenced. The pLLPL2 was confirmed that no changes had happen in the LLPL-2 sequences.

The pLLPL2 was digested with BglII and PmeI and ligated into the BamHI and NruI sites in the Aspergillus expression cassette pCaHj483 which has *Aspergillus niger* neutral amylase promoter, *Aspergillus nidulans* TPI leader sequences, *Aspergillus niger* glucoamylase terminator and *Aspergillus nidulans* amdS gene as a marker. The resultant plasmid was pHUda123.

The LLPL-2 expression plasmid, pHUda123, was digested with NotI and about 6.0 kb DNA fragment containing *Aspergillus niger* neutral amylase promoter, LLPL-2 coding region, *Aspergillus niger* glucoamylase terminator and *Aspergillus nidulans* amdS gene was gel-purified with QIA gel extraction kit.

The 6.0 kb DNA fragment was transformed into *Aspergillus oryzae* BECh-2. The selected transformants were inoculated in 100 ml of MS-9 media and cultivated at 30°C for 1 day. 3 ml of grown cell in MS-9 medium was inoculated to 100 ml of MDU-pH5 medium and cultivated cultivated at 30°C for 4 days.

The supernatant was obtained by centrifugation. The cell was opened by mixed with the equal volume of reaction buffer (50 mM KPB-pH 6.0) and glass-beads for 5 min on ice and debris was removed by centrifugation.

The lysophospholipase productivity of selected transformants was determined as in Example 1. The results shown in the table below clearly demonstrate the absence of increased lysophospholipase activity in supernatants and the presence of increased lysophospholipase activity in cell free extracts.

Strain	Yield (supernatant) Relative activity	Yield (Cell fraction) Relative activity
BECh-2	1.0	1.0
Fg-9	1.0	22.5
Fg-15	1.0	18.0
Fg-27	1.0	17.0
Fg-33	1.0	14.5

Example 3: Cloning and expression of LLPL genes from *E. coli* clones

Each of the following large molecular weight lysophospholipase (LLPL) genes is cloned from the indicated *E. coli* clone as genomic DNA supplier, and the gene is expressed in *A. oryzae* as described in Examples 1 and 2.

Example 5: Identification and sequencing of LLPL-1 and LLPL-2 genes from *A. oryzae*

Cultivation of *A. oryzae*

Aspergillus oryzae strain IFO 4177 was grown in two 20-liter lab fermentors on a 10-liter scale at 34°C using yeast extract and dextrose in the batch medium, and maltose syrup, urea, yeast extract, and trace metals in the feed. Fungal mycelia from the first lab fermentor were harvested by filtering through a cellulose filter (pore size 7-11 microns) after 27 hours, 68.5 hours, 118 hours, and 139 hours of growth. The growth conditions for the second fermentor were identical to the first one, except for a slower growth rate during the first 20 hours of fermentation. Fungal mycelia from the second lab fermentor were harvested as above after 68.3 hours of growth. The harvested mycelia were immediately frozen in liquid N_2 and stored at -80°C.

The *Aspergillus oryzae* strain IFO 4177 was also grown in four 20-liter lab fermentors on a 10-liter scale at 34°C using sucrose in the batch medium, and maltose syrup, ammonia, and yeast extract in the feed. The first of the four fermentations was carried out at pH 4.0. The second of the four fermentations was carried out at pH 7.0 with a constant low agitation rate (550 rpm) to achieve the rapid development of reductive metabolism. The third of the four fermentations was carried out at pH 7.0 under phosphate limited growth by lowering the amount of phosphate and yeast extract added to the batch medium. The fourth of the four fermentations was carried out at pH 7.0 and 39°C. After 75 hours of fermentation the temperature was lowered to 34°C. At 98 hours of fermentation the addition of carbon feed was stopped and the culture was allowed to starve for the last 30 hours of the fermentation. Fungal mycelial samples from the four lab fermentors above were then collected as described above, immediately frozen in liquid N_2, and stored at -80°C.

Aspergillus oryzae strain IFO 4177 was also grown on Whatman filters placed on Cove-N agar plates for two days. The mycelia were collected, immediately frozen in liquid N_2, and stored at -80°C.

Aspergillus oryzae strain IFO 4177 was also grown at 30°C in 150 ml shake flasks containing RS-2 medium (Kofod *et al.*, 1994, *Journal of Biological Chemistry* 269: 29182-29189) or a defined minimal medium. Fungal mycelia were collected after 5 days of growth in the RS-2 medium and 3 and 4 days of growth in the defined minimal medium, immediately frozen in liquid N_2, and stored at -80°C.

Construction of directional cDNA libraries from *Aspergillus oryzae*

Total RNA was prepared by extraction with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion (Chirgwin *et al.*, 1979, *Biochemistry* 18: 5294-5299) using the following modifications. The frozen mycelia were of DEPC-treated water) was heated at 70°C for 8 minutes in a pre-siliconized, RNase-free Eppendorf tube, quenched on ice, and combined in a final volume of 50 il with reverse transcriptase buffer (50 mM Tris-Cl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) containing 1 mM of dATP, dGTP and dTTP, and 0.5 mM of 5-methyl-dCTP, 40 units of human placental ribonuclease inhibitor, 4.81 µg of oligo$(dT)_{18}$-*NotI* primer and 1000 units of SuperScript II RNase H - reverse transcriptase.

First-strand cDNA was synthesized by incubating the reaction mixture at 45°C for 1 hour. After synthesis, the mRNA:cDNA hybrid mixture was gel filtrated through a Pharmacia MicroSpin S-400 HR spin column according to the manufacturer's instructions.

After the gel filtration, the hybrids were diluted in 250 µl of second strand buffer (20 mM Tris-Cl pH 7.4, 90 mM KCl, 4.6 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.16 mM ßNAD$^+$) containing 200 iM of each dNTP, 60 units of *E. coli* DNA polymerase I (Pharmacia, Uppsala, Sweden), 5.25 units of RNase H, and 15 units of *E. coli* DNA ligase. Second strand cDNA synthesis was performed by incubating the reaction tube at 16°C for 2 hours, and an additional 15 minutes at 25°C. The reaction was stopped by addition of EDTA to 20 mM final concentration followed by phenol and chloroform extractions.

The double-stranded cDNA was ethanol precipitated at -20°C for 12 hours by addition of 2 volumes of 96% ethanol and 0.2 volume of 10 M ammonium acetate, recovered by centrifugation, washed in 70% ethanol, dried (SpeedVac), and resuspended in 30 ml of Mung bean nuclease buffer (30 mM sodium acetate pH 4.6, 300 mM NaCl, 1 mM $ZnSO_4$, 0.35 mM dithiothreitol, 2% glycerol) containing 25 units of Mung bean nuclease. The single-stranded hair-pin DNA was clipped by incubating the reaction at 30°C for 30 minutes, followed by addition of 70 ml of 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction, and ethanol precipitation with 2 volumes of 96% ethanol and 0.1 volume 3 M sodium acetate pH 5.2 on ice for 30 minutes.

The double-stranded cDNAs were recovered by centrifugation (20,000 rpm, 30 minutes), and blunt-ended with T4 DNA polymerase in 30 µl of T4 DNA polymerase buffer (20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol) containing 0.5 mM of each dNTP, and 5 units of T4 DNA polymerase by incubating the reaction mixture at +16°C for 1 hour. The reaction was stopped by addition of EDTA to 20 mM final concentration, followed by phenol and chloroform extractions and ethanol precipitation for 12 h at -20°C by adding 2 volumes of 96% ethanol and 0.1 volume of 3M sodium acetate pH 5.2.

After the fill-in reaction the cDNAs were recovered by centrifugation as above, washed in 70% ethanol, and the DNA pellet was dried in a SpeedVac. The cDNA pellet was resuspended in 25 µl of ligation buffer (30 mM Tris-Cl, pH 7.8, 10 ml of 1x TE pH 7.5, loaded on a 0.8% SeaKem agarose gel in 1x TBE, and run on the gel for 3 hours at 60 V. The digested vector was cut out from the gel, and the DNA was extracted from the gel using the GFX gel band purification kit (Amersham-Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. After measuring the DNA concentration by $OD_{260/280}$, the eluted vector was stored at -20°C until library construction.

To establish the optimal ligation conditions for the cDNA library, four test ligations were carried out in 10 ìl of ligation buffer (30 mM Tris-Cl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP) containing 7 µl of double-stranded cDNA, (corresponding to approximately 1/10 of the total volume in the cDNA sample), 2 units of T4 ligase, and 25 ng, 50 ng and 75 ng of *Eco*RI-*Not*I cleaved pYES2.0 vector, respectively (Invitrogen). The vector background control ligation reaction contained 75 ng of *Eco*RI-*Not*I cleaved pYES.0 vector without cDNA. The ligation reactions were performed by incubation at 16°C for 12 hours, heated at 65°C for 20 minutes, and then 10 µl of autoclaved water was added to each tube. One ìl of the ligation mixtures was electroporated (200 W, 2.5 kV, 25 mF) to 40 µl electrocompetent *E. coli* DH10B cells (Life Technologies, Gaithersburg, MD). After addition of 1 ml SOC to each transformation mix, the cells were grown at 37°C for 1 hour, 50 µl and 5 µl from each electroporation were plated on LB plates supplemented with ampicillin at 100 µg per ml and grown at 37°C for 12 hours. Using the optimal conditions, 18 *Aspergillus oryzae* IFO 4177 cDNA libraries containing 1-2.5x10^7 independent colony forming units was established in *E. coli*, with a vector background of ca. 1%. The cDNA library was stored as (1) individual pools (25,000 c.f.u./pool) in 20% glycerol at -80°C; (2) cell pellets of the same pools at -20°C; (3) Qiagen purified plasmid DNA from individual pools at -20°C (Qiagen Tip 100); and (4) directional, double-stranded cDNA at -20°C.

Aspergillus oryzae EST (expressed sequence tag) Template Preparation

From each cDNA library described, transformant colonies were picked directly from the transformation plates into 96-well microtiter dishes (QIAGEN, GmbH, Hilden Germany) which contained 200 µl TB broth (Life Technologies, Frederick Maryland) with 100 µg ampicillin per ml. The plates were incubated 24 hours with agitation (300 rpm) on a rotary shaker. To prevent spilling and cross-contamination, and to allow sufficient aeration, the plates were covered with a microporous tape sheet AirPore™ (QIAGEN GmbH, Hilden Germany). DNA was isolated from each well using the QIAprep 96 Turbo kit (QIAGEN GmbH, Hilden Germany).

dition of 10 ml of 50 °C Cove top agarose, the reaction was poured onto Cove agar plate. Transformation plates were incubated at 32 °C for 5 days.

<u>Expression of LLPL-2 gene in *Aspergillus niger*.</u>

The coding region of the LLPL-2 gene was amplified from genomic DNA of an *Aspergillus niger* strain by PCR with the primers HU225 (SEQ ID NO: 15) and HU226 (SEQ ID NO: 16) which included a BglII and a PmeI restriction enzyme site, respectively.

Reaction components (1 ng /µl of genomic DNA, 250 mM dNTP each, primer 250 nM each, 0.1 U/ µl in Taq polymerase in 1X buffer (Roche Diagnostics, Japan)) were mixed and submitted for PCR under the following conditions.

Step	Temperature	time
1	94 °C	2 min
2	92 °C	1 min
3	55 °C	1 min
4	72 °C	2 min
5	72 °C	10 min
6	4 °C	forever

Step 2 to 4 were repeated 30 times.

The 2 kb fragment was gel-purified with QIA gel extraction kit and ligated into a pT7Blue vector with Ligation high. The ligation mixture was transformed into *E. coli* JM109. The resultant plasmid (pLLPL2) was sequenced, and it was confirmed that no changes had happened in the LLPL-2 sequences.

The pLLPL2 was digested with BglII and PmeI and ligated into the BamHI and NruI sites in the Aspergillus expression cassette pCaHj483 which has *Aspergillus niger* neutral amylase promoter, *Aspergillus nidulans* TPI leader sequences, *Aspergillus niger* glucoamylase terminator and *Aspergillus nidulans* amdS gene as a marker. The resultant plasmid was named pHUda123.

The LLPL-2 expression plasmid, pHUda123, was transformed into an *Aspergillus niger* strain. Selected transformants were inoculated in 100 ml of MLC media and cultivated at 30 °C for 2 days. 5 ml of grown cell in MLC medium was inoculated to 100 ml of MU-1 medium and cultivated at 30 °C for 7 days.

Supernatant was obtained by centrifugation, and the lysophospholipase activity was measured as described above. The table below shows the lysophospholihigh.(TOYOBO). The ligation mixture was transformed into *E. coli* JM109. The resultant plasmid (pHUda126) was sequenced to confirm that nucleotides 115-1824 of SEQ ID NO: 3 were intact and that nucleotides 1825-1914 of SEQ ID NO: 3 had been deleted, corresponding to a C-terminal deletion of amino acids S571-L600 of LLPL-2 (SEQ ID NO: 4)..

The 2.0 kb fragment encoding LLPL-2-CD was obtained by digesting pHUda126 with BglII and SmaI. The 2.0 kb fragment was gel-purified with the QIA gel extraction kit and ligated into the BamHI and NruI sites in the *Aspergillus* expression cassette pCaHj483 with Ligation high. The ligation mixture was transformed into *E. coli* JM109.

The resultant plasmid (pHUda128) for LLPL-2-CD expression cassette was constructed and transformed into the *A. oryzae* strain, BECh-2. Selected transformants were inoculated in 100 ml of MS-9 media and cultivated at 30 °C for 1 day. 3 ml of grown cell in MS-9 medium was inoculated to 100 ml of MDU-pH5 medium and cultivated cultivated at 30 °C for 3 days.

Supernatant was obtained by centrifugation, and the lysophospholipase activity was measured as described above. The table below shows the lysophospholipase activity from of the selected transformants, relative to the activity of the host strain, BECh-2 which was normalized to 1.0.

Strain	Yield (supernatant) Relative activity
BECh-2	1.0
128-3	9
128-9	7
128-12	33
128-15	11

The above results clearly demonstrate the presence of increased lysophospholipase activity in supernatants.

Example 7: Use of *A. niger* LLPL-2 in Filtration

Filtration performance was determined at 60 °C and pH 4.5 using partially hydrolyzed wheat starch, as follows: The wheat starch hydrolyzate (25 ml in a 100 ml flask) was mixed with LLPL-2 from Example 4 at a dosage of 0.4 L/t dry matter and incubated 6 hours at 60 °C under magnetic stirring. A control was made without enzyme addition. After 6 hours incubation the hydrolyzate was decanted into a glass

CLAIMS

1. A lysophospholipase which is:

a) a polypeptide encoded by a lysophospholipase encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* deposit number DSM 13003, DSM 13004, DSM 13082 or DSM 13083, or

b) a polypeptide having an amino acid sequence as the mature peptide shown in SEQ ID NO: 2, 4, 6 or 8, or which can be derived therefrom by substitution, deletion, and/or insertion of one or more amino acids, particularly by deletion of 25-35 amino acids at the C-terminal;

c) an analogue of the polypeptide defined in (a) or (b) which:

i) has at least 70% homology with said polypeptide,

ii) is immunologically reactive with an antibody raised against said polypeptide in purified form, or

iii) is an allelic variant of said polypeptide; or

d) a polypeptide which is encoded by a nucleic acid sequence which hybridizes under high stringency conditions with a complementary strand of the nucleic acid sequence shown as nucleotides 109-1920 of SEQ ID NO: 1, 115-1914 of SEQ ID NO: 3, 70-1881 of SEQ ID NO: 5 or 193-2001 of SEQ ID NO: 7, or a subsequence thereof having at least 100 nucleotides.

2. The lysophospholipase of claim 1 which is native to a strain of *Aspergillus*, preferably *A. niger* or *A. oryzae*.

3. A nucleic acid sequence comprising a nucleic acid sequence which encodes the lysophospholipase of claim 1 or 2.

4. A nucleic acid sequence which comprises:

a) the lysophospholipase encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* DSM 13003, DSM 13004, DSM 13082 or DSM 13083,

b) the nucleic acid sequence shown as nucleotides 109-1920 of SEQ ID NO: 1, 115-1914 of SEQ ID NO: 3, 70-1881 of SEQ ID NO: 5 or 193-2001 of SEQ ID NO: 7,

c) an analogue of the sequence defined in a) or b) which encodes a lysophospholipase and

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gtc att tat ctg cct aac agc ccc tac acc tac gac tcc aac ttc tcg 1584
Val Ile Tyr Leu Pro Asn Ser Pro Tyr Thr Tyr Asp Ser Asn Phe Ser
475 480 485 490

acc ttc aag ctg acc tac agc gac gag gag cgt gat tcc gtc atc acc 1632
Thr Phe Lys Leu Thr Tyr Ser Asp Glu Glu Arg Asp Ser Val Ile Thr
495 500 505

aac ggc tgg aac gtg gtc act cgc ggt aac ggt acc gtt gat gat aac 1680
Asn Gly Trp Asn Val Val Thr Arg Gly Asn Gly Thr Val Asp Asp Asn
510 515 520

ttc ccg tct tgc gtg gcg tgc gct att ctc caa gcg ctc cac tac agg 1728
Phe Pro Ser Cys Val Ala Cys Ala Ile Leu Gln Ala Leu His Tyr Arg
525 530 535

acg aac acc tct ctg cca gat atc tgt acc acc tgc ttt aac gat tac 1776
Thr Asn Thr Ser Leu Pro Asp Ile Cys Thr Thr Cys Phe Asn Asp Tyr
540 545 550

tgc tgg aac ggc acg aca aac agc act acg cct gga gct tat gaa ccc 1824
Cys Trp Asn Gly Thr Thr Asn Ser Thr Thr Pro Gly Ala Tyr Glu Pro
555 560 565 570

agt gtg ctg att gct act agc ggt gcg atc aag agt gtc ttg gat tac 1872
Ser Val Leu Ile Ala Thr Ser Gly Ala Ile Lys Ser Val Leu Asp Tyr
575 580 585

tcg gtg ctg gcg ctc gcc atg ggt gtt gct gcg ttt atg ctg tag 1917
Ser Val Leu Ala Leu Ala Met Gly Val Ala Ala Phe Met Leu
590 595 600

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Met Lys Leu Pro Leu Phe Ala Ala Ala Ala Ala Gly Leu Ala Asn Ala
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Ala Ser Leu Pro Val Glu Arg Ala Glu Ala Glu Val Ala Ser Val Ala
-20 -15 -10

Ala Asp Leu Ile Val Arg Ala Leu Pro Asn Ala Pro Asp Gly Tyr Thr
-5 -1 1 5 10

Pro Ser Asn Val Thr Cys Pro Ser Thr Arg Pro Ser Ile Arg Asp Ala
15 20 25

Ser Gly Ile Ser Thr Asn Glu Thr Glu Trp Leu Lys Val Arg Arg Asn
30 35 40

Ala Thr Leu Thr Pro Met Lys Asn Leu Leu Ser Arg Leu Asn Leu Thr
45 50 55

Gly Phe Asp Thr Thr Ser Tyr Ile Asn Glu His Ser Ser Asn Ile Ser
60 65 70

Asn Ile Pro Asn Ile Ala Ile Ala Ala Ser Gly Gly Gly Tyr Arg Ala
75 80 85 90

Leu Thr Asn Gly Ala Gly Ala Leu Lys Ala Phe Asp Ser Arg Ser Asp
95 100 105

Asn Ala Thr Asn Ser Gly Gln Leu Gly Gly Leu Leu Gln Ala Ala Thr
110 115 120

Tyr Val Ser Gly Leu Ser Gly Gly Ser Trp Leu Val Gly Ser Met Phe
125 130 135

Val Asn Asn Phe Ser Ser Ile Gly Glu Leu Gln Ala Ser Glu Lys Val
140 145 150

Trp Arg Phe Asp Lys Ser Leu Leu Glu Gly Pro Asn Phe Asp His Ile
155 160 165 170

Gln Ile Val Ser Thr Val Glu Tyr Trp Lys Asp Ile Thr Glu Glu Val
175 180 185

Asp Gly Lys Ala Asn Ala Gly Phe Asn Thr Ser Phe Thr Asp Tyr Trp
190 195 200

Gly Arg Ala Leu Ser Tyr Gln Leu Val Asn Ala Ser Asp Asp Lys Gly
205 210 215

Gly Pro Asp Tyr Thr Trp Ser Ser Ile Ala Leu Met Asp Asp Phe Lys
220 225 230

Asn Gly Gln Tyr Pro Met Pro Ile Val Val Ala Asp Gly Arg Asn Pro
235 240 245 250

Gly Glu Ile Ile Val Glu Thr Asn Ala Thr Val Tyr Glu Val Asn Pro
255 260 265

Trp Glu Phe Gly Ser Phe Asp Pro Ser Val Tyr Ala Phe Ala Pro Leu
270 275 280

Gln Tyr Leu Gly Ser Arg Phe Glu Asn Gly Ser Ile Pro Asp Asn Gly
285 290 295

Thr Cys Val Ser Gly Phe Asp Asn Ala Gly Phe Ile Met Gly Ser Ser
300 305 310

Ser Thr Leu Phe Asn Gln Phe Leu Leu Gln Ile Asn Ser Thr Ser Ile
315 320 325 330

Pro Thr Ile Leu Lys Asp Ala Phe Thr Asp Ile Leu Glu Asp Leu Gly
335 340 345

Glu Arg Asn Asp Asp Ile Ala Val Tyr Ser Pro Asn Pro Phe Ser Gly
350 355 360

Tyr Arg Asp Ser Ser Glu Asp Tyr Ala Thr Ala Lys Asp Leu Asp Val
365 370 375

Val Asp Gly Gly Glu Asp Gly Glu Asn Ile Pro Leu His Pro Leu Ile
380 385 390

Gln Pro Glu Arg Ala Val Asp Val Ile Phe Ala Ile Asp Ser Ser Ala
395 400 405 410

Asp Thr Asp Tyr Tyr Trp Pro Asn Gly Thr Ser Leu Val Ala Thr Tyr
415 420 425

Glu Arg Ser Leu Glu Pro Ser Ile Ala Asn Gly Thr Ala Phe Pro Ala
430 435 440

Val Pro Asp Gln Asn Thr Phe Val Asn Leu Gly Leu Asn Ser Arg Pro
445 450 455

Thr Phe Phe Gly Cys Asp Pro Lys Asn Ile Ser Gly Thr Ala Pro Leu
460 465 470

Val Ile Tyr Leu Pro Asn Ser Pro Tyr Thr Tyr Asp Ser Asn Phe Ser

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475                 480                 485                 490

Thr Phe Lys Leu Thr Tyr Ser Asp Glu Glu Arg Asp Ser Val Ile Thr
            495                 500                 505

Asn Gly Trp Asn Val Val Thr Arg Gly Asn Gly Thr Val Asp Asp Asn
        510                 515                 520

Phe Pro Ser Cys Val Ala Cys Ala Ile Leu Gln Ala Leu His Tyr Arg
    525                 530                 535

Thr Asn Thr Ser Leu Pro Asp Ile Cys Thr Thr Cys Phe Asn Asp Tyr
540                 545                 550

Cys Trp Asn Gly Thr Thr Asn Ser Thr Thr Pro Gly Ala Tyr Glu Pro
555                 560                 565                 570

Ser Val Leu Ile Ala Thr Ser Gly Ala Ile Lys Ser Val Leu Asp Tyr
            575                 580                 585

Ser Val Leu Ala Leu Ala Met Gly Val Ala Ala Phe Met Leu
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Met Lys Val Ala Leu Leu Thr Leu Ala Ala Gly Leu Ala Asn Ala Ala
        -20                 -15                 -10

tcg atc gcc gtc act cca cgg gcg ttc ccc aat gcc cct gat aaa tat        96
Ser Ile Ala Val Thr Pro Arg Ala Phe Pro Asn Ala Pro Asp Lys Tyr
    -5                  -1  1               5

gct ccc gca aat gtt tcc tgt ccg tcg act cgt ccc agt atc cgc agt       144
Ala Pro Ala Asn Val Ser Cys Pro Ser Thr Arg Pro Ser Ile Arg Ser
10                  15                  20                  25
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gcc gcc gcc ctg tcc acc agt gag aag gat tgg ttg caa gtg cgt cgg 192
Ala Ala Ala Leu Ser Thr Ser Glu Lys Asp Trp Leu Gln Val Arg Arg
30 35 40

aat gag acc ctt gaa ccc atg aag gat ttg ctc ggg cgg ctc aat cta 240
Asn Glu Thr Leu Glu Pro Met Lys Asp Leu Leu Gly Arg Leu Asn Leu
45 50 55

agc tcc ttt gat gcc tcg ggg tac att gac cgt cat aaa aac aat gca 288
Ser Ser Phe Asp Ala Ser Gly Tyr Ile Asp Arg His Lys Asn Asn Ala
60 65 70

tcg aat att cca aac gtg gcc att gcc gtt tca ggt ggt ggt tac cgc 336
Ser Asn Ile Pro Asn Val Ala Ile Ala Val Ser Gly Gly Gly Tyr Arg
75 80 85

gct ttg acc aat ggc gcg ggt gct atc aag gca ttc gat agt cgt acc 384
Ala Leu Thr Asn Gly Ala Gly Ala Ile Lys Ala Phe Asp Ser Arg Thr
90 95 100 105

tcc aac tcc aca gcc cgt gga cag ctc gga ggc ctt ctg cag tcc tct 432
Ser Asn Ser Thr Ala Arg Gly Gln Leu Gly Gly Leu Leu Gln Ser Ser
110 115 120

act tat cta tcg ggc ctc agt ggt ggt gga tgg ctc gtg ggc tcc gtg 480
Thr Tyr Leu Ser Gly Leu Ser Gly Gly Gly Trp Leu Val Gly Ser Val
125 130 135

tac atc aac aac ttc acc act atc ggt gac ctg cag gcc agc gac aag 528
Tyr Ile Asn Asn Phe Thr Thr Ile Gly Asp Leu Gln Ala Ser Asp Lys
140 145 150

gtc tgg gac ttc aag aac tct att ctg gag ggt cct gat gtt aaa cat 576
Val Trp Asp Phe Lys Asn Ser Ile Leu Glu Gly Pro Asp Val Lys His
155 160 165

ttc caa ctg atc aac act gcc gcg tac tgg aag gat ctg tac gat gcg 624
Phe Gln Leu Ile Asn Thr Ala Ala Tyr Trp Lys Asp Leu Tyr Asp Ala
170 175 180 185

gtg aag gat aag aga aac gcc ggg ttc aac act tcg ttg acc gac tac 672
Val Lys Asp Lys Arg Asn Ala Gly Phe Asn Thr Ser Leu Thr Asp Tyr
190 195 200

tgg ggc cgt gct ctc tcc tat cag ttc atc aac gct acc act gat gat 720
Trp Gly Arg Ala Leu Ser Tyr Gln Phe Ile Asn Ala Thr Thr Asp Asp
205 210 215

ggc ggt ccc agt tat acc tgg tcg tcg att gcc ttg ggc gac gat ttc 768
Gly Gly Pro Ser Tyr Thr Trp Ser Ser Ile Ala Leu Gly Asp Asp Phe
220 225 230

aag aag ggc aag atg ccc atg cct atc ctc gtc gcc gat gga cgt aac 816
Lys Lys Gly Lys Met Pro Met Pro Ile Leu Val Ala Asp Gly Arg Asn
235 240 245

ccg ggc gaa ata ctt att gga agt aac tcg act gtg tat gaa ttt aac 864
Pro Gly Glu Ile Leu Ile Gly Ser Asn Ser Thr Val Tyr Glu Phe Asn
250 255 260 265

cca tgg gag ttc ggc tcc ttc gac ccg tca gta tac ggc ttt gca cca 912

Pro Trp Glu Phe Gly Ser Phe Asp Pro Ser Val Tyr Gly Phe Ala Pro
270 275 280

ttg gag tat ctt gga tcc aat ttc gag aac ggt gaa ctc ccc aag ggg 960
Leu Glu Tyr Leu Gly Ser Asn Phe Glu Asn Gly Glu Leu Pro Lys Gly
285 290 295

gaa tcg tgc gtg cgc ggc ttt gac aat gcg ggt ttt gtc atg ggt acc 1008
Glu Ser Cys Val Arg Gly Phe Asp Asn Ala Gly Phe Val Met Gly Thr
300 305 310

agc tct tcc ctg ttt aac cag ttc att ctg cgt ctg aac ggc acc gat 1056
Ser Ser Ser Leu Phe Asn Gln Phe Ile Leu Arg Leu Asn Gly Thr Asp
315 320 325

atc cct aat ttc ctc aag gag gcg att gcc gac gtc ttg gaa cat ctg 1104
Ile Pro Asn Phe Leu Lys Glu Ala Ile Ala Asp Val Leu Glu His Leu
330 335 340 345

ggc gaa aac gat gag gac att gca gtt tac gca ccc aac ccc ttc tac 1152
Gly Glu Asn Asp Glu Asp Ile Ala Val Tyr Ala Pro Asn Pro Phe Tyr
350 355 360

aaa tat cgc aat tca acg gca gca tat tcg tca acc cca gag ctg gac 1200
Lys Tyr Arg Asn Ser Thr Ala Ala Tyr Ser Ser Thr Pro Glu Leu Asp
365 370 375

gtg gtc gac gga ggt gaa gat gga cag aac gtg cct cta cac ccg ttg 1248
Val Val Asp Gly Gly Glu Asp Gly Gln Asn Val Pro Leu His Pro Leu
380 385 390

atc cag ccc acc cac aac gtg gat gtg atc ttt gcc gtg gat tcg tcc 1296
Ile Gln Pro Thr His Asn Val Asp Val Ile Phe Ala Val Asp Ser Ser
395 400 405

gct gat acg gac cat agc tgg ccc aac gga tcc tcc ttg atc tac acc 1344
Ala Asp Thr Asp His Ser Trp Pro Asn Gly Ser Ser Leu Ile Tyr Thr
410 415 420 425

tat gaa cgt agc ttg aat act aca ggt atc gcc aac ggg acc tcc ttc 1392
Tyr Glu Arg Ser Leu Asn Thr Thr Gly Ile Ala Asn Gly Thr Ser Phe
430 435 440

cct gcg gtg ccc gac gtc aac acg ttc ctc aac ctt ggc ctg aac aaa 1440
Pro Ala Val Pro Asp Val Asn Thr Phe Leu Asn Leu Gly Leu Asn Lys
445 450 455

cgc ccg acc ttc ttc gga tgc aat tca tcc aac acc agc acc ccg acc 1488
Arg Pro Thr Phe Phe Gly Cys Asn Ser Ser Asn Thr Ser Thr Pro Thr
460 465 470

cca ttg att gtc tac ttg ccc aac gcc cct tac acc gcc gag tcc aac 1536
Pro Leu Ile Val Tyr Leu Pro Asn Ala Pro Tyr Thr Ala Glu Ser Asn
475 480 485

acg tca acc ttc cag ctg gcg tat aag gac caa caa cgc gat gat att 1584
Thr Ser Thr Phe Gln Leu Ala Tyr Lys Asp Gln Gln Arg Asp Asp Ile
490 495 500 505

atc ttg aac ggc tac aac gtc gtc acc cag ggc aat gcc agt gcc gat 1632
Ile Leu Asn Gly Tyr Asn Val Val Thr Gln Gly Asn Ala Ser Ala Asp

510 515 520

gca aac tgg ccc tcg tgc gtt ggg tgc gct att ctc cag cgg tcc acc 1680
Ala Asn Trp Pro Ser Cys Val Gly Cys Ala Ile Leu Gln Arg Ser Thr
525 530 535

gaa cgt acg aac act aag ctt ccc gat atc tgc aat acc tgc ttc aag 1728
Glu Arg Thr Asn Thr Lys Leu Pro Asp Ile Cys Asn Thr Cys Phe Lys
540 545 550

aat tac tgc tgg gac gga aag acc aac agc acc aca ccg gcc ccc tat 1776
Asn Tyr Cys Trp Asp Gly Lys Thr Asn Ser Thr Thr Pro Ala Pro Tyr
555 560 565

gaa ccg gag cta ttg atg gag gcg tcg act tcc ggg gcc tcg aag gat 1824
Glu Pro Glu Leu Leu Met Glu Ala Ser Thr Ser Gly Ala Ser Lys Asp
570 575 580 585

caa ctg aac cgg aca gct gca gtc atc gcg ttc gca gtt atg ttc ttt 1872
Gln Leu Asn Arg Thr Ala Ala Val Ile Ala Phe Ala Val Met Phe Phe
590 595 600

atg acg atc tag 1884
Met Thr Ile

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Ser Ile Ala Val Thr Pro Arg Ala Phe Pro Asn Ala Pro Asp Lys Tyr
-5 -1 1 5

Ala Pro Ala Asn Val Ser Cys Pro Ser Thr Arg Pro Ser Ile Arg Ser
10 15 20 25

Ala Ala Ala Leu Ser Thr Ser Glu Lys Asp Trp Leu Gln Val Arg Arg
30 35 40

Asn Glu Thr Leu Glu Pro Met Lys Asp Leu Leu Gly Arg Leu Asn Leu
45 50 55

Ser Ser Phe Asp Ala Ser Gly Tyr Ile Asp Arg His Lys Asn Asn Ala
60 65 70

Ser Asn Ile Pro Asn Val Ala Ile Ala Val Ser Gly Gly Gly Tyr Arg
75 80 85

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Ala Leu Thr Asn Gly Ala Gly Ala Ile Lys Ala Phe Asp Ser Arg Thr
90                  95                  100                 105

Ser Asn Ser Thr Ala Arg Gly Gln Leu Gly Gly Leu Leu Gln Ser Ser
            110                 115                 120

Thr Tyr Leu Ser Gly Leu Ser Gly Gly Gly Trp Leu Val Gly Ser Val
        125                 130                 135

Tyr Ile Asn Asn Phe Thr Thr Ile Gly Asp Leu Gln Ala Ser Asp Lys
    140                 145                 150

Val Trp Asp Phe Lys Asn Ser Ile Leu Glu Gly Pro Asp Val Lys His
155                 160                 165

Phe Gln Leu Ile Asn Thr Ala Ala Tyr Trp Lys Asp Leu Tyr Asp Ala
170                 175                 180                 185

Val Lys Asp Lys Arg Asn Ala Gly Phe Asn Thr Ser Leu Thr Asp Tyr
            190                 195                 200

Trp Gly Arg Ala Leu Ser Tyr Gln Phe Ile Asn Ala Thr Thr Asp Asp
        205                 210                 215

Gly Gly Pro Ser Tyr Thr Trp Ser Ser Ile Ala Leu Gly Asp Asp Phe
    220                 225                 230

Lys Lys Gly Lys Met Pro Met Pro Ile Leu Val Ala Asp Gly Arg Asn
235                 240                 245

Pro Gly Glu Ile Leu Ile Gly Ser Asn Ser Thr Val Tyr Glu Phe Asn
250                 255                 260                 265

Pro Trp Glu Phe Gly Ser Phe Asp Pro Ser Val Tyr Gly Phe Ala Pro
            270                 275                 280

Leu Glu Tyr Leu Gly Ser Asn Phe Glu Asn Gly Glu Leu Pro Lys Gly
        285                 290                 295

Glu Ser Cys Val Arg Gly Phe Asp Asn Ala Gly Phe Val Met Gly Thr
    300                 305                 310

Ser Ser Ser Leu Phe Asn Gln Phe Ile Leu Arg Leu Asn Gly Thr Asp
315                 320                 325
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Ile Pro Asn Phe Leu Lys Glu Ala Ile Ala Asp Val Leu Glu His Leu
330 335 340 345

Gly Glu Asn Asp Glu Asp Ile Ala Val Tyr Ala Pro Asn Pro Phe Tyr
350 355 360

Lys Tyr Arg Asn Ser Thr Ala Ala Tyr Ser Ser Thr Pro Glu Leu Asp
365 370 375

Val Val Asp Gly Gly Glu Asp Gly Gln Asn Val Pro Leu His Pro Leu
380 385 390

Ile Gln Pro Thr His Asn Val Asp Val Ile Phe Ala Val Asp Ser Ser
395 400 405

Ala Asp Thr Asp His Ser Trp Pro Asn Gly Ser Ser Leu Ile Tyr Thr
410 415 420 425

Tyr Glu Arg Ser Leu Asn Thr Thr Gly Ile Ala Asn Gly Thr Ser Phe
430 435 440

Pro Ala Val Pro Asp Val Asn Thr Phe Leu Asn Leu Gly Leu Asn Lys
445 450 455

Arg Pro Thr Phe Phe Gly Cys Asn Ser Ser Asn Thr Ser Thr Pro Thr
460 465 470

Pro Leu Ile Val Tyr Leu Pro Asn Ala Pro Tyr Thr Ala Glu Ser Asn
475 480 485

Thr Ser Thr Phe Gln Leu Ala Tyr Lys Asp Gln Gln Arg Asp Asp Ile
490 495 500 505

Ile Leu Asn Gly Tyr Asn Val Val Thr Gln Gly Asn Ala Ser Ala Asp
510 515 520

Ala Asn Trp Pro Ser Cys Val Gly Cys Ala Ile Leu Gln Arg Ser Thr
525 530 535

Glu Arg Thr Asn Thr Lys Leu Pro Asp Ile Cys Asn Thr Cys Phe Lys
540 545 550

Asn Tyr Cys Trp Asp Gly Lys Thr Asn Ser Thr Thr Pro Ala Pro Tyr
555 560 565

Glu Pro Glu Leu Leu Met Glu Ala Ser Thr Ser Gly Ala Ser Lys Asp

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570                 575                 580                 585

Gln Leu Asn Arg Thr Ala Ala Val Ile Ala Phe Ala Val Met Phe Phe
                590                 595                 600

Met Thr Ile


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cgaccacttc aggtcagt atg aaa ccc aca aca gct gca att gct tta gcc    111
                    Met Lys Pro Thr Thr Ala Ala Ile Ala Leu Ala
                            -35                 -30

ggg ttg ctg tct ggc gtg aca gcg gcc cca ggc cct cat gga gaa agg    159
Gly Leu Leu Ser Gly Val Thr Ala Ala Pro Gly Pro His Gly Glu Arg
    -25                 -20                 -15

att gag agg att gat aga act gtg ttg gaa cgt gca ttg cca aat gct    207
Ile Glu Arg Ile Asp Arg Thr Val Leu Glu Arg Ala Leu Pro Asn Ala
-10                 -5              -1  1               5

ccc gat gga tat gta ccg tcc aac gtc agt tgt cct gcg aat cgc ccg    255
Pro Asp Gly Tyr Val Pro Ser Asn Val Ser Cys Pro Ala Asn Arg Pro
            10                  15                  20

acg gtg cgt agc gca tca tcc ggg ctc tcg agc aat gag acc tcg tgg    303
Thr Val Arg Ser Ala Ser Ser Gly Leu Ser Ser Asn Glu Thr Ser Trp
        25                  30                  35

ttg aaa acc cga cgg gag aag act caa tct gcc atg aaa gat ttc ttc    351
Leu Lys Thr Arg Arg Glu Lys Thr Gln Ser Ala Met Lys Asp Phe Phe
    40                  45                  50

aac cat gtc acg att aag gac ttt gat gct gtc caa tat ctc gac aac    399
Asn His Val Thr Ile Lys Asp Phe Asp Ala Val Gln Tyr Leu Asp Asn
55                  60                  65

cac tcg agt aac acg tcc aat ctt ccc aat att ggt att gcg gtg tct    447
His Ser Ser Asn Thr Ser Asn Leu Pro Asn Ile Gly Ile Ala Val Ser
70                  75                  80                  85

ggt gga ggt tat cgc gcc ctg atg aac ggt gcc gga gcg atc aaa gcg    495
Gly Gly Gly Tyr Arg Ala Leu Met Asn Gly Ala Gly Ala Ile Lys Ala
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            90                  95                  100

ttt gat agc cga acg gag aac tcg acg gcg acg gga cag ttg ggt ggt    543
Phe Asp Ser Arg Thr Glu Asn Ser Thr Ala Thr Gly Gln Leu Gly Gly
        105                 110                 115

ctg cta cag tcg gcg acg tat ctg gct ggt ctg agt ggt ggt gga tgg    591
Leu Leu Gln Ser Ala Thr Tyr Leu Ala Gly Leu Ser Gly Gly Gly Trp
    120                 125                 130

ctg gtg ggg tcg atc tat atc aac aat ttc acc acc att tca gca ctg    639
Leu Val Gly Ser Ile Tyr Ile Asn Asn Phe Thr Thr Ile Ser Ala Leu
135                 140                 145

cag acc cat gag gat ggt gct gtc tgg cag ttt caa aac tcg att ttt    687
Gln Thr His Glu Asp Gly Ala Val Trp Gln Phe Gln Asn Ser Ile Phe
150                 155                 160                 165

gag ggc cct gac ggc gat agc att cag att ctg gat tct gcg act tac    735
Glu Gly Pro Asp Gly Asp Ser Ile Gln Ile Leu Asp Ser Ala Thr Tyr
            170                 175                 180

tac aag cac gtt tac gat gca gtg caa gac aag aag gat gcg gga tac    783
Tyr Lys His Val Tyr Asp Ala Val Gln Asp Lys Lys Asp Ala Gly Tyr
        185                 190                 195

gaa acc tct atc act gat tat tgg ggt cgc gct ctc tct tat caa tta    831
Glu Thr Ser Ile Thr Asp Tyr Trp Gly Arg Ala Leu Ser Tyr Gln Leu
    200                 205                 210

atc aat gct acc gac ggc ggt ccg agc tat act tgg tcg tcc att gcc    879
Ile Asn Ala Thr Asp Gly Gly Pro Ser Tyr Thr Trp Ser Ser Ile Ala
215                 220                 225

cta acc gat aca ttt aag cag gca gat atg ccg atg cct ctc ctc gtt    927
Leu Thr Asp Thr Phe Lys Gln Ala Asp Met Pro Met Pro Leu Leu Val
230                 235                 240                 245

gcc gac ggt cgg tat ccc gat gag ctc gtg gtc agc agc aac gct act    975
Ala Asp Gly Arg Tyr Pro Asp Glu Leu Val Val Ser Ser Asn Ala Thr
            250                 255                 260

gtc tat gag ttt aac cct tgg gag ttt ggt act ttt gat cca aca gtc    1023
Val Tyr Glu Phe Asn Pro Trp Glu Phe Gly Thr Phe Asp Pro Thr Val
        265                 270                 275

tac ggg ttt gtg cct cta gaa tac gta ggc tct aaa ttc gac ggt ggt    1071
Tyr Gly Phe Val Pro Leu Glu Tyr Val Gly Ser Lys Phe Asp Gly Gly
    280                 285                 290

tct atc ccc gac aac gag acc tgt gta cgc gga ttc gac aac gcc ggt    1119
Ser Ile Pro Asp Asn Glu Thr Cys Val Arg Gly Phe Asp Asn Ala Gly
295                 300                 305

ttt gtt atg ggt act tcg tca agt ttg ttc aac cag ttc ttc ctg cag    1167
Phe Val Met Gly Thr Ser Ser Ser Leu Phe Asn Gln Phe Phe Leu Gln
310                 315                 320                 325

gtt aac tca act tcg ctt cct gat ttc ctg aag acg gca ttc tcg gac    1215
Val Asn Ser Thr Ser Leu Pro Asp Phe Leu Lys Thr Ala Phe Ser Asp
            330                 335                 340
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atc ttg gca aag att ggt gaa gaa gat gag gac att gct gtc tat gca      1263
Ile Leu Ala Lys Ile Gly Glu Glu Asp Glu Asp Ile Ala Val Tyr Ala
        345                 350                 355

ccc aac ccg ttc tac aat tgg gcc ccc gtg agc tca cca gca gcc cat      1311
Pro Asn Pro Phe Tyr Asn Trp Ala Pro Val Ser Ser Pro Ala Ala His
    360                 365                 370

caa cag gaa ctc gat atg gtg gac ggt ggc gag gat ctt cag aac att      1359
Gln Gln Glu Leu Asp Met Val Asp Gly Gly Glu Asp Leu Gln Asn Ile
375                 380                 385

cct ctg cat cct tta att cag cca gag cgt cac gta gat gtt atc ttt      1407
Pro Leu His Pro Leu Ile Gln Pro Glu Arg His Val Asp Val Ile Phe
390                 395                 400                 405

gct gtt gac tcc tcc gcc gac acg act tat tct tgg ccc aac ggc aca      1455
Ala Val Asp Ser Ser Ala Asp Thr Thr Tyr Ser Trp Pro Asn Gly Thr
                410                 415                 420

gct ctc gtt gcc act tac gag cgc agc ctg aac tcc acc ggc atc gct      1503
Ala Leu Val Ala Thr Tyr Glu Arg Ser Leu Asn Ser Thr Gly Ile Ala
            425                 430                 435

aac gga acc tca ttc ccc gcg atc cct gac cag aat acc ttt gtt aac      1551
Asn Gly Thr Ser Phe Pro Ala Ile Pro Asp Gln Asn Thr Phe Val Asn
        440                 445                 450

aat ggc ttg aat acg cgg cca acg ttc ttc gga tgt aac agt acg aac      1599
Asn Gly Leu Asn Thr Arg Pro Thr Phe Phe Gly Cys Asn Ser Thr Asn
    455                 460                 465

acc aca ggc cct acg cct ttg gtt gtc tac ctt ccg aac tat cca tac      1647
Thr Thr Gly Pro Thr Pro Leu Val Val Tyr Leu Pro Asn Tyr Pro Tyr
470                 475                 480                 485

gtg tct tac tcg aac tgg tca acc ttc cag cca agc tat gag atc tcc      1695
Val Ser Tyr Ser Asn Trp Ser Thr Phe Gln Pro Ser Tyr Glu Ile Ser
                490                 495                 500

gaa aga gac gac acc atc cgc aac gga tat gat gtg gtg acg atg ggt      1743
Glu Arg Asp Asp Thr Ile Arg Asn Gly Tyr Asp Val Val Thr Met Gly
            505                 510                 515

aac agc act cgt gat ggt aac tgg acg acc tgc gtc ggt tgt gct att      1791
Asn Ser Thr Arg Asp Gly Asn Trp Thr Thr Cys Val Gly Cys Ala Ile
        520                 525                 530

ctg agt cgg tct ttc gag cgc acg aac acc cag gtt ccg gat gcc tgc      1839
Leu Ser Arg Ser Phe Glu Arg Thr Asn Thr Gln Val Pro Asp Ala Cys
    535                 540                 545

acc cag tgc ttc cag aag tac tgc tgg gat ggc act acg aac tcc acc      1887
Thr Gln Cys Phe Gln Lys Tyr Cys Trp Asp Gly Thr Thr Asn Ser Thr
550                 555                 560                 565

aac cct gcc gac tat gag cct gtc acc ctg ttg gag gat agt gct ggt      1935
Asn Pro Ala Asp Tyr Glu Pro Val Thr Leu Leu Glu Asp Ser Ala Gly
                570                 575                 580
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tcc gct ctc tcc ccg gct gtc atc acc acc atc gta gcg acc agt gct 1983
Ser Ala Leu Ser Pro Ala Val Ile Thr Thr Ile Val Ala Thr Ser Ala
585 590 595

gct ctt ttc acc ttg ctg tgagactgga gcaattctgt tggatacggc 2031
Ala Leu Phe Thr Leu Leu
600

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Lys Asp Phe Asp Ala Val Gln Tyr Leu Asp Asn His Ser Ser Asn Thr
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Ser Asn Leu Pro Asn Ile Gly Ile Ala Val Ser Gly Gly Gly Tyr Arg
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Ala Leu Met Asn Gly Ala Gly Ala Ile Lys Ala Phe Asp Ser Arg Thr
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Glu Asn Ser Thr Ala Thr Gly Gln Leu Gly Gly Leu Leu Gln Ser Ala

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Tyr Ile Asn Asn Phe Thr Thr Ile Ser Ala Leu Gln Thr His Glu Asp
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Gly Ala Val Trp Gln Phe Gln Asn Ser Ile Phe Glu Gly Pro Asp Gly
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Asp Ser Ile Gln Ile Leu Asp Ser Ala Thr Tyr Tyr Lys His Val Tyr
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Gly Gly Pro Ser Tyr Thr Trp Ser Ser Ile Ala Leu Thr Asp Thr Phe
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Pro Asp Glu Leu Val Val Ser Ser Asn Ala Thr Val Tyr Glu Phe Asn
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Pro Trp Glu Phe Gly Thr Phe Asp Pro Thr Val Tyr Gly Phe Val Pro
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Tyr Glu Arg Ser Leu Asn Ser Thr Gly Ile Ala Asn Gly Thr Ser Phe
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Pro Ala Ile Pro Asp Gln Asn Thr Phe Val Asn Asn Gly Leu Asn Thr
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Arg Pro Thr Phe Phe Gly Cys Asn Ser Thr Asn Thr Thr Gly Pro Thr
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Trp Ser Thr Phe Gln Pro Ser Tyr Glu Ile Ser Glu Arg Asp Asp Thr
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20

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 00/00577

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/16 C12N15/63 //(C12N9/16,C12R1:685,C12R1:69)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MASUDA N ET AL: "Primary structure of protein moiety of Penicillium Notatum phospholipase B deduced from the cDNA" EUR J BIOCHEM, vol. 202, 1991, pages 783-787, XP002901491 -& DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; MASUDA N ET AL: "Primary structure of protein moiety of Pencillium Notatum phospholipase B deduced from the cDNA" retrieved from MEDLINE, accession no. 92111525 Database accession no. P39457 XP002901492 62.9% identity in 614 aa overlap abstract --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 2001	08.03.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Yvonne Siösteen

1

Form PCT/ISA/210 (second sheet) (July 1992)

NZAS-0238954

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 00/00577

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

ɔrmation on patent family members

Intern nal Application No
PCT/DK 00/00577

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9831790 A	23-07-1998	DE 19701348 A	23-07-1998
		AU 6208098 A	07-08-1998
		BR 9805893 A	24-08-1999
		CA 2243476 A	23-07-1998
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		CN 1216061 T	05-05-1999
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US 5965422 A	12-10-1999	DE 19620649 A	27-11-1997
		AU 718990 B	04-05-2000
		AU 1997697 A	27-11-1997
		CA 2205411 A	22-11-1997
		EP 0808903 A	26-11-1997
US 6146869 A	14-11-2000	NONE	